ITC Tutorials

I. Introduction.

These experimental tutorials are designed by MicroCal to acquaint you with the basic features of both the hardware and software of the VP-ITC instrument, as well as to provide experience with several manipulations, which must be mastered in order to get the highest quality data from your instrument. Rather than beginning experimentation on the VP-ITC using precious biological samples, we strongly suggest that each user of the VP-ITC instrument complete the following tutorial first, using methanol solutions, so that irreplaceable samples are not wasted while mastering the appropriate techniques.

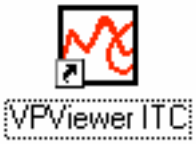
II. Precautions.

A. When using volatile or hazardous solutions in the cells, follow proper laboratory procedures in handling and disposing of such materials (e.g. wear safety glasses and protective clothing) and allow the internal cells to cool down to room temperature before removing any solutions from the cells.
B. All solutions in the cells should be cooled down below 50 ºC before removing from the cells to prevent the glass syringes from breaking due to the hot liquids.
C. Avoid materials that corrode the Hastelloy® Alloy C-276 cells. Hastelloy is extremely resistant to corrosion by strong bases, but strong acids should be avoided.
D. Prevent freezing solutions in the cells. The freezing of solutions in filled cells will cause irreparable damage to the cells due to the expansion of the solution in the cells. If operations below 0 °C are required, the cells should contain a non-freezing solution or at most 250 µl (half filled) of a solution that could freeze.

III. Clean the cells according the "Cell Cleaning Procedure" and "How To Use The Thermovac" documents.

IV. Ensure that Instrument and PC are turned on.

A. When powering up the VP-ITC, always follow the sequence listed immediately below.
    1. Turn on PC power.
    2. Turn on VP-ITC power at the left top rear of the unit.
    3. Launch VPViewer. The VPViewer2000 window pops up in front of the Origin window.



V. Sample Preparation. Methanol Tutorial.

NOTE: During degassing, you can set up the run parameters.

A. Degas (with stirring) ~40 ml of distilled water for ~7 minutes with the ThermoVac. This degassed water will be the sample (and also reference cell if it does not already contain water).
B. To ~20 ml of the degassed water in a test tube, 0.5 ml add methanol (0.5 ml) to make a 2.5% methanol solution.

C. Degas the 2.5% methanol solution for 5 minutes, with stirring. This solution will be loaded into the injection syringe later.

VI. Filling the Cells

A. Remove the cap of the calorimeter chamber.

B. Load the 2.5-ml glass filling syringe with water as follows:

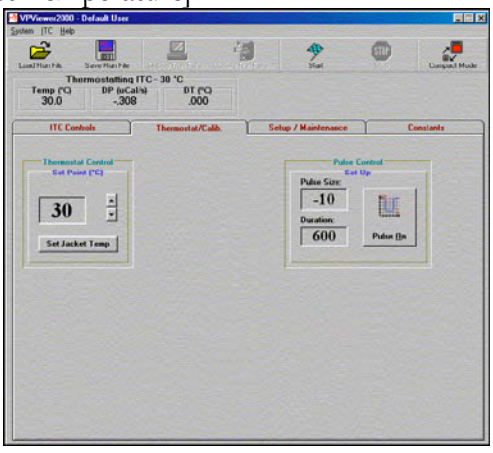
1. Hold the syringe needle-up and tap the syringe bottom so all bubbles float to the top surface.
2. Enter the needle into the sample cell entry tube (this is the center hole located to the right of the reference entry hole). Carefully slide the needle down the tube until it just touches the bottom of the cell.
3. Lift the syringe so that the needle end is just off bottom (less than 1 mm).
4. Slowly depress the plunger so that the cell fills from the bottom up.
5. After ~1.7 ml of the degassed water has been introduced into the cell, the water will be seen coming to the top of the small entry tube.
6. The liquid level rises above the top of the access tube, depress the plunger very quickly 1-3 times to deliver abrupt bursts of 0.3 ml. The purpose of these last bursts is to dislodge any bubbles, which might be clinging where the entry tube joins the cell.

C. If the reference cell has not previously been filled with degassed water, fill it in the same manner.

1. There is no need to refill the reference cell with each experiment. A water reference may be good for a week or two with no attention if the water was thoroughly degassed before filling.
2. To prevent sample overflow into the reference cell, a small stopper and insertion device has been provided to cap the reference cell access tube after filling.

D. Check the VP-ITC thermostat temperature and make sure it is set to the desired run temperature of 30 degrees. If the current thermostat temperature is not set to 30 degrees, set it now as follows:

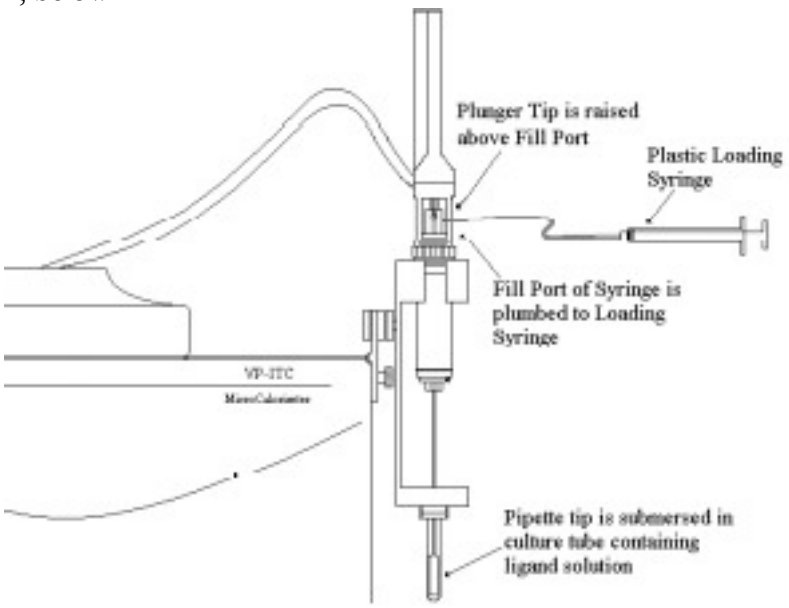
1. Select the "Thermostat/Calib." tab on the VPViewer2000 window.
2. Enter the temperature on the left of the page.
3. Click [Set Jacket Temperature].



VII. Load the Pipette.

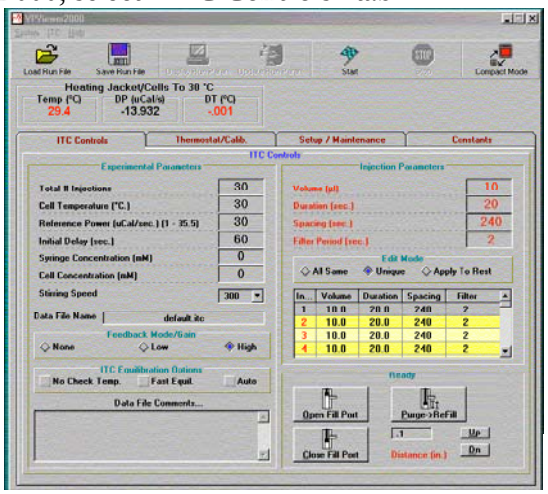
A. Degas the titrant for 5 minutes and place it in the pipette filling culture tube.

B. Screw the sample loading culture tube into the bottom of the pipette stand. Refer to the diagram, below.


Plunger Tip is raised above Fill Port
Plastic Loading Syringe
Fill Port of Syringe is plumbed to Loading Syringe
VP-ITC
Pipette tip is submersed in culture tube containing ligand solution

C. Carefully insert the pipette into the pipette stand.

1. The paddle on the end of the syringe should not hit the bottom or the sides of the sample tube.
2. Pipette loading will be easiest when the tip of the syringe paddle is just slightly above the bottom of the sample tube.
3. Open the fill port as follows:
4. From VPViewer2000, select "ITC Controls" tab.



5. Click on the [Open Fill Port] button at the lower right of the window. The plunger will move until the Teflon tip is just above the filling port of the syringe.

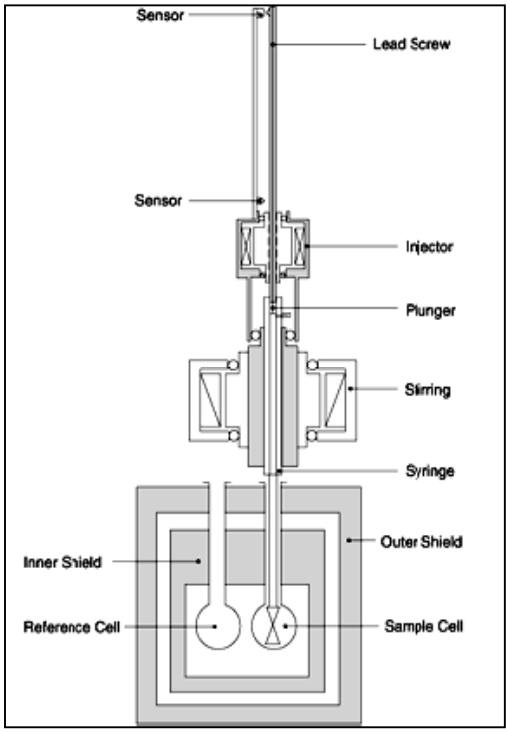
Wait until the movement is complete.

D. Attach the tube of the plastic filling syringe to the filling port of the injection syringe. Refer the to above diagram.
E. Slowly withdraw the plastic syringe plunger to draw up the titrant solution until you see solution exit through the top filling port into the syringe tubing.
F. Click on the [Close Fill Port] button.
   1. The pipette will lower the plunger of the injection syringe until the white Teflon tip is completely below the filling port (ca. 4mm).
   2. Wait until the movement is complete.
G. Remove the hose and the plastic filling syringe.
H. Click on the [Purge->ReFill] button.
   1. The pipette will depress the plunger of the filling syringe to inject the sample back into the filling test tube, then raise the plunger to refill the injection syringe.
   2. Wait until the movement in done. The plunger tip will be just below the filling port. A complete movement takes about 35 seconds.
I. Click on the [Purge !ReFill] button, again to dislodge any air bubbles from inside walls of the injection syringe and expel them back into the titrant solution.

VIII. Place Syringe Into Sample Cell.

! Caution: Be careful not to hit the long needle of the injection syringe against any object. This could bend the needle and expel some solution from the syringe resulting in a poor first injection for the experiment. If the long needle is bent enough, then it may be permanently deformed and unusable for future experiments.

A. Ensure that the cap is removed from the calorimeter chamber.
B. Carefully remove the pipette from its stand and wipe the tip and shaft.
C. Carefully insert the pipette into the sample cell access tube on the right.
D. While watching the paddle to insure it is inserted directly into the access hole, hold the pipet vertically and slowly lower the pipet into the cell. You may have to push down slightly to overcome the resistance of the rubber o-ring.

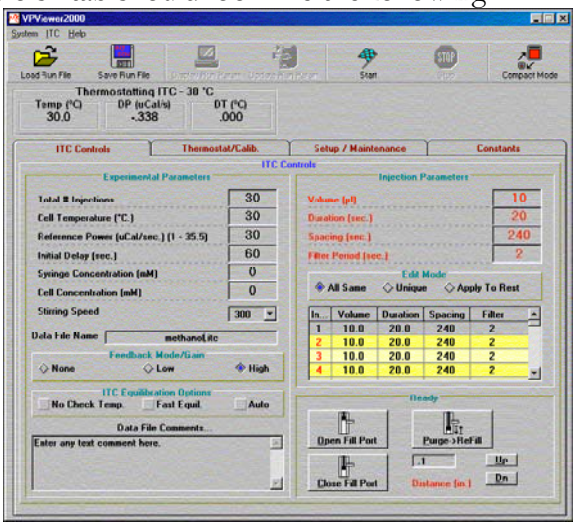


IX. Enter Run Parameters in VPViewer.

A. Load the run parameters for the methanol dilution experiment as follows:
   1. Press the [Load Run File] button.
   2. Open **methanol.inj** to load the run parameters.
   3. The Experimental Parameters are as follows:

*Total # Injections = 6-11.
*Cell Temperature = 30 °C.
*Reference Power = 30 µCal/s.
*Initial Delay = 60 s.
*Syringe Concentration = 0 (for this tutorial).
*Cell Concentration = 0 (for this tutorial).
*Stirring Speed = 300.
*Data File Name = [Enter a unique name that you will remember].
*Feedback Mode/Gain = High.
*Remove all check marks for the "ITC Equilibration Options" to observe each step of the equilibration process. Normally, you may want a check next to "Auto" to start the titration experiment without operator attention.

4. The Injection Parameters are as follows:
   *Volume = 10 µl.
   *Duration = 20 s.
   *Spacing = 240 s.
   *Filter Period = 2 s.
   *Edit Mode = "All Same".
5. The "ITC Controls" tab should look like the following:



6. Press [Start] from the VPViewer2000 window. The cell will eventually reach the set temperature.
7. Press [Compact Mode] to reduce the size of the VPViewer2000 acquisition window.

X. Pre-Stirring Equilibration.

A. Observe the **Pre-Stirring Equilibration** data being plotted versus time in the VPViewer2000.
   1. Three different Y-axis scales with four different data sets will be plotted in the graph.
      a) Each Y-axis is plotted in its own layer (see Origin User's Manual for

more information about layers).

b) Notice the 3-layer button located in the upper left corner of the graph. The active layer is a black number while the inactive layers have white numbers.

2. For this tutorial we entered 30 for the Baseline Position, you may see that there may be an initial decrease in the DP power (due to the added frictional energy due to stirring), but then the DP values will increase because of the power applied to the reference which forces the feedback system to apply power to the sample cell to compensate and maintain a temperature balance.
3. Set a layer as active by clicking on the layer button or the corresponding Y-axis. The Y-axis on the left is colored blue and the data set plotted in this layer (also colored blue) is the DP data (DP data is the differential power between the reference and sample cell). When Prestirring equilibration is first started the data may not be visible on the graph.

B. Click on the [Auto-View 2] button to center the current data point with a Y-axis full scale of 10 (ten).

C. Click on the [Auto-View 1] button to more closely view the data.

1. If you switch to another application while displaying data in VPViewer then switch back to VPViewer a gap in the trace may appear. These data are not lost. Select [Window] [Refresh] to plot the missing data.
2. When the graph first appears, the DP data display box in VPViewer's main window has the values displayed in red.

D. Wait for the cell to equilibrate in the **Pre-stirring mode.** The VPViewer2000 DP (μCal/s) data display will turn green indicating that the cell has equilibrated in the **Pre-stirring mode**. Note that the text in the Origin plot window will remain red.

E. Manually start the Stirring equilibration by double-clicking on the green VPViewer2000 DP (μCal/s) data display. This display will turn red, again.

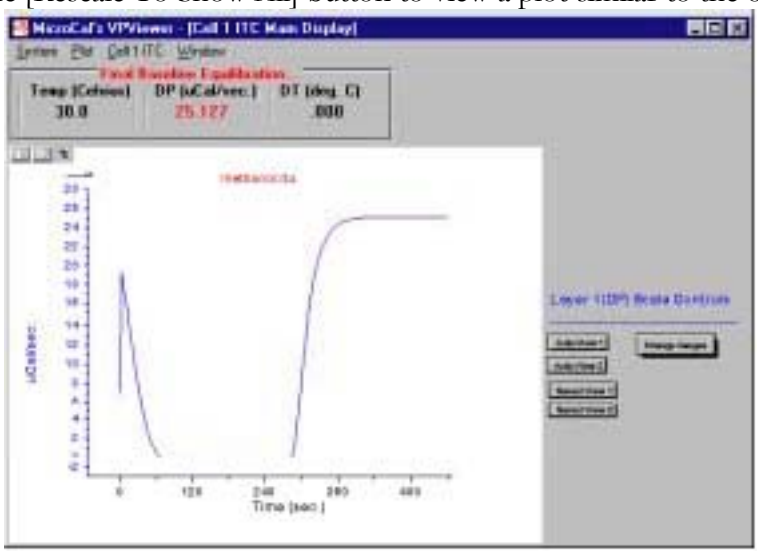
XI. Final Baseline Equilibration.

A. When the Pre-stirring baseline has stabilized, the display will turn green. Double-click in the DP display box to start the Final Baseline Equilibration. The data display will turn red, and the display title will now read Final Baseline Equilibration.

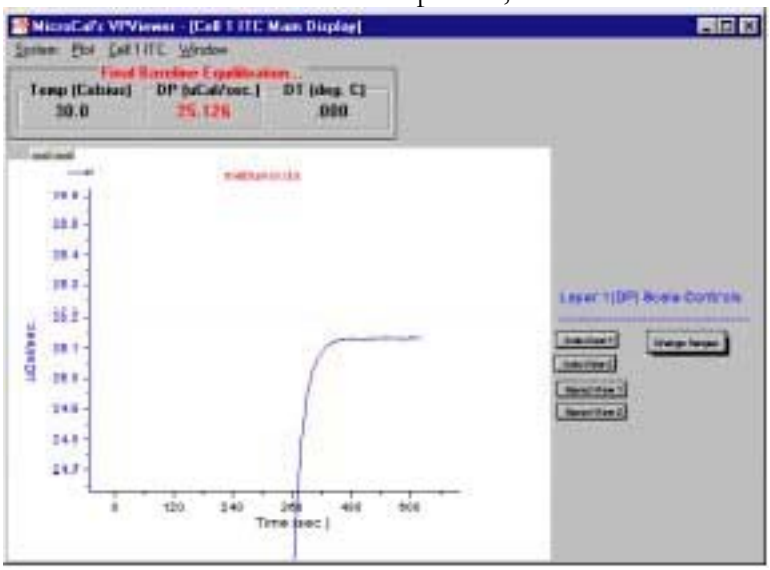
B. When VPViewer starts the **Final Baseline Equilibration**, the stirring starts and power is applied to the reference cell.

1. The amount of power applied to the reference cell is determined by what you entered into the Baseline Position (μCal/sec) text box located in the Experimental Parameters group of the Cell 1 (ITC) Run Parameters dialog box.
2. For this tutorial we entered 30 for the Baseline Position, you may see that there may be an initial decrease in the DP power (due to the added frictional energy due to stirring), but then the DP values will increase because of the power applied to the reference which forces the feedback system to apply power to the sample cell to compensate and maintain a temperature balance.
3. The effect of this heat disturbance will take a few minutes for a new equilibration point with the final DP values being close to 25.

C. Click on the [Rescale To Show All] button to view a plot similar to the one below.



D. To take a closer look at the final baseline before proceeding, click on the [Auto-View 1] button. (Note: You can also double click on the X-axis and enter new limits for the X-axis). You should see a view similar to the picture, below.



E. Wait for the Final Baseline to equilibrate. The display will turn green. If [Auto] was checked, then VPViewer would have automatically started the experiment after the display turned green.

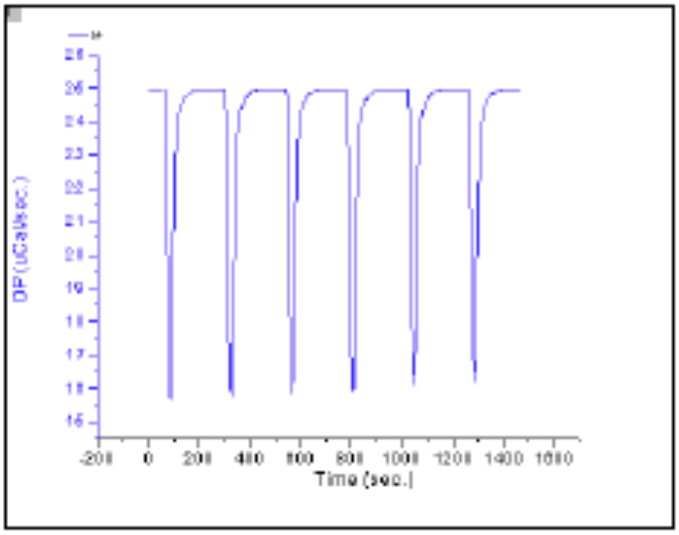
XII. Start the Experiment

A. Double-click anywhere in the DP display box, to start the experiment.
   1. The DP display box will turn black.
   2. The display title will read "Pre-Titration Delay".

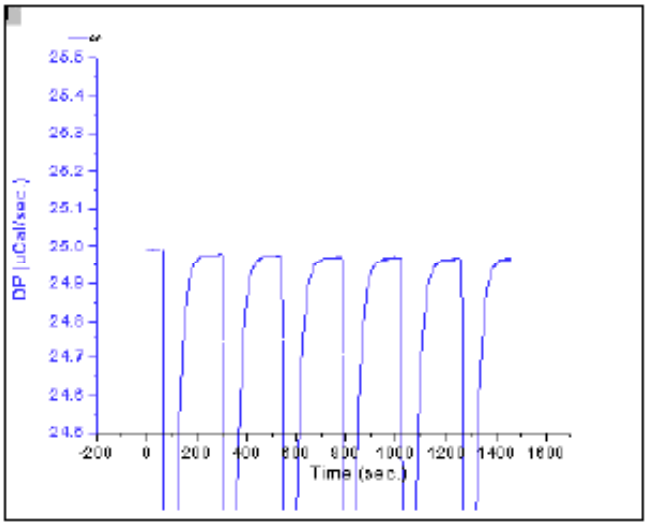
3. The volume of solution that is left in the syringe will be displayed.
4. The **Pre-Titration Delay** is required for an initial baseline for the first injection of the experiment.

XIII. Observe the Experiment.

A. After a few injections, view the injection peaks.
B. Click on [Rescale to Show All] to see a plot similar to the one below.



C. Click on [Auto View 1] button to get a closer look at the baseline.

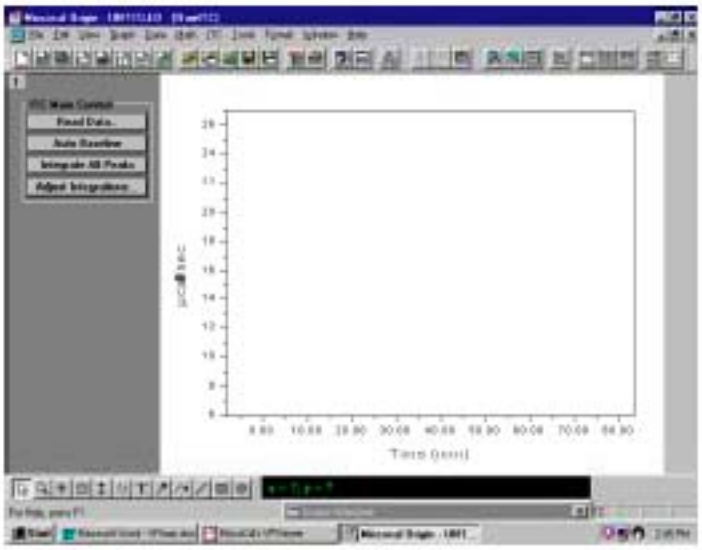


D. Use a y-axis display range of 1 µCal/sec. and an x-axis display range of 30 minutes (1800 sec.).

XIV. Plot and Analyze MicroCal's Data in Origin.

A. While your experiment continues, minimize both the VPViewer and Origin applications.

B. Double-click the "Microcal Inc ITC" icon on the desktop to launch another instance of Origin. The raw data template appears.



C. Click on the [Read Data] button to import your VP-ITC data into the plot window.
   1. Browse to and open c:\vpitc\data\methanol.itc. This data files was generated at MicroCal on an VP-ITC instrument using 10 ul injections of a 2.5% methanol solution. Your peak sizes may differ a little.

D. Click on the plot or select DeltaH from the Window menu to make it active.

E. Click on the layer icon box, a gray box with '1' appearing in it located in the far upper left. The layer control dialogue box for the DeltaH plot will open.

F. Move the methanol_ndh file out of the Layer Contents (click on the name methanol_ndh then click on the ['] button) and move the methanol_ndh file from Available Data to Layer Contents (click on methanol_dh then click on the [!] button).

G. Click [OK]. The new data will be plotted as a line.

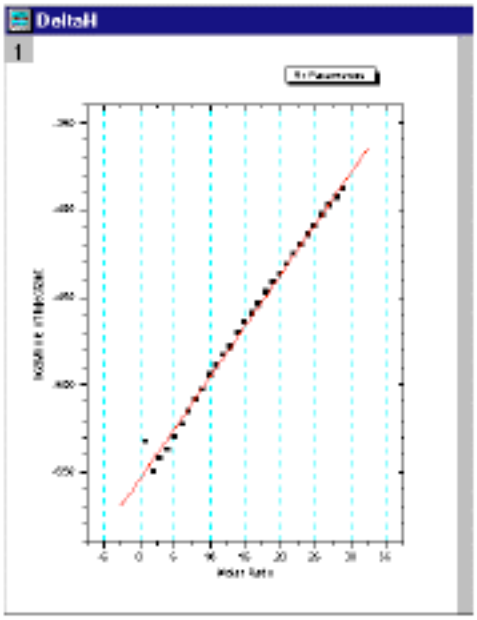
H. To change this plot symbol, right-click on the plot line and select [Change to Scatter] from the drop down menu list.

I. To change to kcal from cal, double-click on the y-axis. A dialogue box for the y-axis tick labels pops up.
   1. Select the "Tick Labels" tab.
   2. In the Factor box, change Divide by Factor to 1.
   3. Click OK. The data set is plotted in terms of kcal per injection on the y-axis vs. a

number proportional to injection number on the x-axis.

J. To remove the undesired first data point, click on [Remove Bad Data].
   1. Click on the data point to be removed.
   2. Press the [Enter] key to remove it.

K. Compare your results with those shown. Your data will have 5-10 data points. The deviation from a straight line should not be much worse than these. A linear regression fit can be obtained by selecting Math | Linear Regression. Or, you may integrate all of the peaks one-by-one, rather than relying on the automatic integration procedure. This might reduce the scatter.



L. Again, compare your results to the plot. One can expect to receive similar heats. Most importantly will be the linearity of the peak heights and of the peak areas.

M. If the data with 2.5% methanol looks considerably worse than the sample data, then repeat this tutorial. Also, review the Troubleshooting section in the MicroCal manual.

N. When your experiment is done, analyze it in the same manner. If you are satisfied with your methanol dilution data, then move on to your samples.

XV. RNase A - 2'CMP Binding Tutorial and Data Processing. This is just an example for references purposes. This tutorial will not be performed.

MicroCal used this experiment to generate a binding isotherm. If your techniques are good, you should be able now to generate the RNase-2'CMP binding isotherm and obtain fitting parameters similar to those obtained at MicroCal. If everything has gone well to this point, then you should be ready to begin studies on your own samples.

A. Materials.
   1. 0.057 mM RNase (Sigma R5500) in 50 mM potassium acetate, pH 5.5 @ 30°C.
   2. 2.16 mM 2'-CMP (Sigma C7137) in 50 mM potassium acetate, pH 5.5 @ 30°C.

B. Procedure.
   1. Acquisition Parameters.
      *Number of Injections: 16
      *Run Temperature: 30
      *Reference Power: 10
      *Initial Delay: 60
      *Syringe Concentration: (enter the concentration).
      *Cell Concentration: (enter the concentration).
      *Stirring Speed: 460

*No Check Temp.: True
*Fast Equil.: True
*Auto: True
*Injection duration = 11.86 s.
*Inject at (default) min/injection.
*Inject 3 µl for the first 4 injections and 6 µl for each subsequent injection.
*Use a 2 s filter.

2. Fill the 250 µl syringe with the 2'-CMP solution and begin the run.

C. Process the Results.

1. Launch Origin ("Microcal Inc ITC") icon from the desktop.
2. Click on [Read Data] and open the file, C:\Origin50\samples\Rnahhh.itc. The RNAHHH data are plotted as a line graph in the RawITC window in units of µcal/second vs. minutes. The following operations are performed automatically.
   a) Auto Baseline routine is selected where each injection peak is analyzed and a baseline is created.
   b) Integrate All Peaks routine is selected where the peaks are integrated and the area (µcal) under each peak is obtained.
   c) The DeltaH window is opened where the normalized area data, rnahhh_ndh, in kcal per mole of injectant versus the molar ratio ligand/macromolecule are plotted. Note that the DeltaH window contains buttons that access ITC routines.
3. Double click on the layer icon (grey number in upper left) to view the available data sets. Eight data sets are created. (Two temporary data sets are also created; rnahhhbegin contains the indices (row numbers) of the beginning of an injection; rnahhhrange contains the indices of the integration range for the injection.). The naming convention for the 8 data sets are shown below, that is. The name of the ITC source file will be followed by an identifying suffix (injection number is indicated by the row number *i*).

rnahhh_dh	Experimental heat change resulting from injection *i*, in µcal/injection (not displayed).
rnahhh_mt	Concentration of macromolecule in the cell *before* each injection *i*, after correction for volume displacement (not displayed).
rnahhh_xt	Concentration of injected solute in the cell *before* each injection (not displayed).
rnahhh_injv	Volume of injectant added for the injection *i*.
rnahhh_ndh	Normalized heat change for injection *i*, in calories per mole of injectant added (displayed in DeltaH window).
rnahhh_xmt	Molar ratio of ligand to macromolecule after injection *i*.
rnahhhbase	Baseline for the injection data (displayed in red in the RawITC window).
rnahhhraw_cp	All of the original injection data (displayed in black in the RawITC window).

4. Three worksheets that hold these data sets are also created. These worksheets will be described later.
5. Save the area data (the RNAHHH integration results) as a separate data file to use this data later to subtract reference data.
    a) Select Window:DeltaH to make DeltaH the active window. Alternatively, you may press and hold the Ctrl key and press the tab key to scroll through Origin's open windows.
    b) Click on the Save Area Data button.
    c) Select a folder for the file and click OK.
6. Ensure that the concentration values are correct by clicking on the [Concentration] button. Close the box when you are done. For the MicroCal RNase data set, use the following:
    a) Syringe concentration = 2.16 mM 2'-CMP.
    b) Cell concentration = 0.057 mM RNase.
    c) Cell volume = 1.42 ml.
7. Remove the first bad data point.
8. Fit the data. Origin provides three built-in curve fitting models: One Set of Sites, Two Sets of Sites, and Sequential Binding Sites.
    a) Click on the [One Set of Sites] button. The Fitting Function Parameters dialog box opens with initial values for the three fitting parameters for this model - N, K, and H.
    b) The fitting parameters are initialized and an initial fit curve is plotted as a straight line, in red.
    c) Click either the [1 Iter.] or [10 Iter.] button to control the iteration of the fitting cycles. It is usually necessary that the 10 Iter. command be used more than once before a good fit is achieved. Repeat this step until you are satisfied with the fit, and Chi^2 is no longer decreasing. Note that the fitting parameters in the dialog box update reflect the current fit.
    d) Fitting Parameter Constraints
    e) Each fitting model has a unique set of fitting parameters. For the One Set of Sites model these are N (number of sites), K (binding constant in M^{-1}), and ΔH (heat change in cal/mole). A fourth parameter, ΔS (cal/mole/deg), is calculated from ΔH and K. You can use the Fitting Session dialog box to apply mathematical constraints to the fitting parameters.
9. Results from Microcal for the RNase 2'-CMP experiment are as follows:
    a) Model = OneSite
    b) Chi^2 = 4291.20
    c) N 0.9564 0.001363
    d) K 6.599E4 1.103E4
    e) ΔH-1.783E436.28
    f) ΔS -32.20

D. Print the results by selecting File | Print. You may have to move (drag with cursor) the results box so that it does not overlap the plot. Ensure that the A/B box is set to "B".

E. Save the project under File | Save.
F. For a detailed discussion and more advanced fitting methods, please refer to the manufacturers documentation, *ITC Data Analysis in Origin*® and the Origin® manuals.

II. Shutdown.

A. Power down the ITC.
B. Clean the syringe as follows:
    1. Place the syringe into its holder.
    2. Place a 5% soluiton Contrad-70 into the syringe cell.
    3. Open the fill hole.
    4. Attach the tubing and syrings to the port.
    5. Draw up the solution through the syringe.
    6. Rinse well with a copious amount of deionized water in the same manner.
C. Clean the cells per *How To Use The ThermoVac* and *Calorimeter Cell Cleaning Procedure.*
D. Power down the computer and ThermoVac.
E. Log out of the logbook.